Fecal microbiota transplantation from high caloric-fed donors alters glucose metabolism in recipient mice, independently of adiposity or exercise status.

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Abstract

Studies suggest the gut microbiota contributes to the development of obesity and the metabolic syndrome. Exercise alters microbiota composition and diversity and is protective of these maladies. We tested whether the protective metabolic effects of exercise are mediated through fecal components through assessment of body composition and metabolism in recipients of fecal microbiota transplantation (FMT) from exercise-trained (ET) mice fed normal or high energy diets. Donor C57BL/6J mice were fed a chow or high fat, high sucrose (HFHS) diet for 4wk to induce obesity and glucose intolerance. Mice were divided into sedentary (Sed) or ET groups [6wk treadmill-based ET] while maintaining their diets, resulting in four donor groups; chow sedentary (NC-Sed) or ET (NC-ET) and HFHS sedentary (HFHS-Sed) or ET (HFHS-ET). Chow-fed recipient mice were gavaged with feces from the respective donor groups weekly, creating four groups (NC-Sed-R, NC-ET-R, HFHS-Sed-R, HFHS-ET-R) and body composition and metabolism assessed. The HFHS diet led to glucose intolerance and obesity in the donors, while exercise training (ET) restrained adiposity and improved glucose tolerance. No donor group FMT altered recipient body composition. Despite unaltered adiposity, glucose levels were disrupted when challenged in mice receiving feces from HFHS-fed donors, irrespective of donor-ET status, with a decrease in insulin-stimulated glucose clearance into white adipose tissue and large intestine and specific changes in the recipient's microbiota composition observed. FMT can transmit HFHS-induced disrupted glucose metabolism to recipient mice independently of any change in adiposity. However, the protective metabolic effect of ET on glucose metabolism is not mediated through fecal factors.

INTRODUCTION

The gut microbiome is a large and sophisticated community of bacteria, fungi and archaea that reside within the gastrointestinal tract (GIT). It has become clear that the composition of this community is associated with various pathological conditions. As this community plays a role in host metabolism, alterations to its membership, distribution or activity may impact metabolic-related disorders such as obesity, insulin resistance and type 2 diabetes (T2D) (13, 21, 29, 32, 33, 41). The microbiota state can be influenced by many factors, particularly host dietary composition and intake pattern. Other environmental and lifestyle factors such as medication, exposure to pollutants, sleep and physical activity are also known microbiota modulators. Indeed, numerous studies in humans and animal models have reported microbiota changes upon exercise, and in athletes (2, 3, 5, 6, 10, 12, 20, 26, 27, 30, 34, 35, 39). Yet, the precise beneficial or negative effects of exercise-induced microbiota alteration to the host remain largely unknown.

Exercise training (ET) is widely accepted as a therapeutic intervention and protective against the development of obesity, insulin resistance and T2D (7). However, many individuals, especially the injured, frail and/or elderly, cannot exercise on a regular basis. Of those who can, rates of compliance are low. Therefore, identifying therapeutics that target pathways regulated by exercise and/or identifying modes of exercise giving maximal metabolic improvement are of interest. With exercise conferring many metabolic benefits through numerous mechanisms, it is unclear (and indeed difficult to discern) whether the microbiota is a conduit for such exercise-induced benefits.

93 Fecal microbiota transplantation (FMT) strives to engineer improvements in gut function
94 and/or systemic health through the introduction of crude stool preparations from healthy
95 donors. It has proven successful in treating chronic *Clostridium difficile* infections (40).
96 Experimentally, FMT can reveal whether given effects are mediated through fecal
97 components and are thusly transmittable. From a metabolic perspective, FMT has
98 demonstrated some metabolism-related phenotypes to be fecal component-modulated in
99 experimental models and human pilot trials, however the corresponding mechanistic
100 foundations remain unknown (9, 23, 24, 37). FMT effects are rarely apportioned between
101 fecal components of live cells, microbial cell components or metabolic factors of either
102 microbe or host origin. The host is sensitive to all these components (36), and we postulate
103 that any of them could exert a regulatory effect on metabolism.
104
105 Here, we assessed whether the metabolic benefits exercise confers on the host are mediated
106 through fecal factors. Specifically, we assessed whether transfer of feces from exercise-
107 trained (ET) or sedentary donor mice, on either a normal or high caloric diet, alters
108 microbiota composition, body composition and glucose metabolism in sedentary, normal
109 chow-fed recipients. Our use of recipients on a chow diet was to provide the greatest
110 opportunity for microbiota transfer to elicit an effect without the confounding factor of a
111 microbiota-modulating high-energy diet in the recipients. We hypothesized that recipients of
112 FMT from high fat, high sucrose (HFHS)-fed, sedentary donor mice (HFHS-Sed) would have
113 increased adiposity and disrupted glucose tolerance compared to mice receiving FMT from
114 mice fed a chow diet. Further, we hypothesized that compared to mice that were sedentary,
115 ET in the donors would protect the metabolic profile of recipients when they were
116 transplanted with fecal matter from HFHS-fed mice. While the FMT was able to transmit
117 HFHS-induced microbiota changes and disrupt glucose metabolism in recipient mice

118 independently of any change in adiposity, FMT from ET donor mice elicited no protective
119 effect on glucose metabolism.

MATERIALS AND METHODS

Animals

C57BL/6J mice were sourced from Alfred Medical Research and Education Precinct (AMREP) Animal Services. All animals were fed a normal chow diet (NC) (14.0MJ/kg, 75.2% kJ from carbohydrate, 4.8% from fat, 20% from protein: Specialty Feeds, WA, Australia) until they initiated the studies at 7-8 wk of age. Thereafter, depending on their allocated group, they were fed NC or a high fat, high sucrose diet (HFHS) (19MJ/kg, 36% kJ from carbohydrate (17% sucrose), 43% from fat 21% from protein; Specialty Feeds, WA, Australia) until study endpoint. The NC diet had a 5.2% total crude fibre composition and the HFHS diet 5.4%. Food and water access were unrestricted (except for experimental fasting periods). Mice were maintained at 22±1°C on a 12h light/dark cycle. The study was approved by the AMREP Animal Ethics Committee and conducted in accordance with National Health and Medical Research Council of Australia guidelines. Experimental procedure flowcharts (11) are provided in Figs 1-2.

Study design

The study involved both recipient and donor groups of male mice run in parallel (See Fig 3). Two cohorts were used to limit experimental burden per mouse and only one sex to remove the confounding physiological gender differences in this initial study. C57BL/6J mice were bred in two rounds, first producing pups for the donor groups and thereafter for recipient groups. At weaning, mice were re-housed across cages to remove cage-mate gut microbiome correlations (12) as a confounding factor. After 4 weeks of NC or HFHS diet (a time frame we have previously demonstrated induces full glucose intolerance and insulin resistance from HFHS diet (42)), donor mice commenced 6 weeks of treadmill running ET (or Sed control); NC or HFHS allocations were retained throughout. Four donor groups resulted: NC-

Sedentary (NC-Sed), NC-Exercise-trained (NC-ET), HFHS-Sedentary (HFHS-Sed) and HFHS-Exercise-trained (HFHS-ET). At both initiation of diet and exercise interventions mouse body weights were analysed to exclude biases resulting from randomised group allocations (Fig 4A, E). Feces were collected from each donor mouse once weekly during the ET period for FMT.

Recipient mice were NC-fed and sedentary. Groups were randomly assigned to receive FMT from each of the four donor groups: NC-Sedentary recipient (NC-Sed-R), NC-Exercise-trained recipient (NC-ET-R), HFHS-Sedentary recipient (HFHS-Sed-R) and HFHS-exercise-trained recipient (HFHS-ET-R). Recipient baseline body weight analysis discounted any randomisation-induced bias (Fig 4A). Mice were housed only with mice receiving the same treatment and received one gavage of fecal slurry per week for 6-weeks and metabolic parameters were assessed.

Fecal Microbiota Transplantation (FMT)

One freshly voided stool per donor mouse was collected each week and combined with other stools from the same group. Stool pellets were sliced with a scalpel and resuspended in 1ml phosphate-buffered saline (PBS) per stool. Stools were homogenised, then twice vortexed and incubated for 15-minutes at 37^0C, then vortexed again and spun at 800rpm for 3-minutes. The crude aqueous fecal extract was oral gavaged to recipients (200µl each) based on previous protocols (43). By performing weekly collections and gavages as the exercise training program progressed, we were attempting to simulate likely changes in the microbiota population over the course of a training program and transferring these to the recipients to assess any impact on metabolic read-outs.

Exercise Capacity Test

An acute incremental exercise test of donors was performed at commencement and completion of the ET intervention. Mice performed a 3-day familiarization protocol in which intensity and durations of treadmill running (Model Exer-3/6 Treadmill, Columbus Instruments, OH, USA) were progressively increased. The test began at 10m/min for 3 min. The velocity was increased by 4m/min every 3 min until fatigue. This was defined as spending >10 sec at the base of the treadmill despite manual encouragement.

Exercise training

Each session consisted of interval training, alternating 2 min of active running with 2 min of rest. Each session lasted for 60 min repeated 3 times per week. The initial speed was 16.0m/min. Each week the speed was increased by 1m/min as progressive overload. Sedentary mice were removed from their holding room and their cages placed next to the treadmill for the duration of the running to control for the activity of removing them from their environment. To control for any acute exercise effects, training was withheld for at least 48 h prior to glucose tolerance testing.

Body composition analysis

Fat mass and lean mass were measured with a 4-in-1 EchoMRI body composition analyzer (EchoMRI^{TM}, TX, USA) and standard laboratory scales were used for total body mass (Mettler Toledo, Greifensee, Switzerland) as previously described in full (28).

Oral glucose tolerance test

Oral glucose tolerance tests (oGTT) were performed on fasted (6 h) mice. Mice received an oral gavage of 2g glucose/kg lean body mass (25% glucose solution) and blood glucose levels were measured via a glucometer (AccuCheck, NSW, Australia) at the indicated times on blood that was collected from the tail.

Metabolic caging analysis (CLAMS)

A Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, OH, USA) was utilized to measure various aspects of metabolism as previously described (28). Mice were individually housed and oxygen consumption (VO_2), respiratory exchange ratio (RER), Energy expenditure (heat) and total movement (beam breaks) were recorded over a 48 h period. The first 24hrs served as an acclimatisation period and the 24-48h period was analysed.

***In vivo* intestinal permeability**

As a marker of gut permeability, a 500mg/kg bolus (125mg/ml) of Fluorescein isothiocyanate–conjugated dextran (FITC-labelled dextran, Sigma-Aldrich, MO, USA) was administered by oral gavage to fasting mice. Blood was obtained from the tail into a heparinised capillary tube and plasma read on a fluorescent plate reader (Ex 490mm, Em 520mm).

Intravenous insulin tolerance test (ivITT)

Intravenous insulin tolerance tests (ivITT) with combined glucose tracer were performed as previously described (17). Briefly, mice were anesthetized and their jugular vein cannulated. Following basal glucose measurements, a single bolus injection of insulin (0.6U/kg lean body mass) also containing [^{3}H]2-deoxyglucose (10μCi) (PerkinElmer, MA, USA) was injected down the line. Blood was sampled at 0, 2, 5, 10, 20 and 30 min for determination of blood

glucose and 2, 10, 20 and 30 min for plasma radioactivity of [^{3}H]2-DG. At endpoint, the organs were excised and snap frozen and stored at -80°C.

Determination of plasma and tissue radioactivity

The collected blood samples (10 µl) were deproteinized with barium hydroxide and zinc sulfate liquid scintillation fluid added and [^{3}H]2-DG radioactivity determined. Accumulation of [^{3}H]2-DG radioactivity in the tissue samples was determined by scintillation counting in an aqueous extract of the tissue after a homogenisation process. Free and phosphorylated [^{3}H]2-DG were separated by ion exchange chromatography on Dowex 1-X8 columns. The area under the tracer disappearance curve for [^{3}H]2-DG and the radioactivity for the phosphorylated [^{3}H]2-DG from the organs were used to calculate the tissue-specific glucose clearance Kg′ as previously described (8, 18).

Fecal DNA extraction and sequencing

DNA was extracted from the feces using a FastDNA Spin Kit for feces (MP Biomedicals, CA, USA) and sequencing conducted using a 16S V1-3 (27f/519r) amplicon on an Illumina MiSeq v3 at the Ramaciotti Centre for Genomics, Sydney, Australia.

Microbial ecology profiling

Microbial ecology analyses were performed using R; all analysis code is publicly available at https://github.com/marknormanread/henstridge-2019. Raw sequence reads were clustered into amplicon sequence variants (ASV) with inferred taxonomies using the 'DADA2' R package (4). Microbiota composition, alpha diversity, and sequencing depth analyses were performed with the 'phyloseq' R package (31). Alpha diversity and sequencing depth statistical comparisons were performed using PERMANOVA ('adonis' function from the 'vegan' R package (19)). Rarefaction analysis was performed using custom code. Principal

component analyses (PCA) were performed using the 'mixomics' R package (38); data were transformed using the isometric logratio transformation. Isometric and centred logratio transformations reduce biases inherent in the compositional nature of microbial sequencing data (15). Microbes *Bacteriodales S24-7* were renamed *Bacteriodales Muribaculaceae* in accordance with recent characterizations thereof (25).

Statistical significance of microbiota clusterings by experimental group

This analysis was performed with custom code. We employ the Aitchison metric (Euclidean distance between centred logratio transformed data) to calculate the pairwise distance between all samples. Thereafter, the distributions of within-group and between-group sample distances are contrasted using a one-sided Kolmogorov-Smirnov statistic; if the latter exceeds the former, then the groups have statistically significantly different microbiota compositions. P values were corrected for multiple comparisons using the Bonferroni method.

Identifying microbial signatures through supervised machine learning

Models were trained that predict experimental groups from microbiota profiles; the taxa employed by models form microbial signatures of experimental interventions. We employed the 'leave-one-out' cross validation (LOO-CV) methodology to build predictive models with square discriminant analysis (sPLS-DA), using the 'mixomics' R package (38). The available data is repeatedly partitioned into a 'building' portion used in model construction and a 'validation' portion used to assess model performance. Under LOO-CV, each sample is retained as the sole validation portion member exactly once, with all remaining samples forming the building portion; hence, all available data are ultimately used (once) in assessing model performance. Model predictions of validation sample 'class' membership (experimental group) are through mahalanobis distance. Classification accuracies are reported for each class individually. Because the number of samples differs across experimental

groups, models were trained to minimise the balanced error rate (the mean average error rate across classes; each class is equally important regardless of size) rather than the overall error rate (% of total errors, biased towards larger classes). The error rate is 1-accuracy. Microbial ecology sequencing data is typically noisy and encompasses more numerous taxa than samples, which together risk spurious associations of taxa with experimental groups. Our methodology mitigates this risk by constructing models on the 'building' portions of repeated bifurcations of our data and uses these models to predict the experimental group of the withheld samples. The noise in building and validation portions differs and, hence, well-performing microbial signatures likely represent genuine signal and not noise.

The statistical significance of sPLS-DA model classification accuracy is estimated through permutation testing (reported in Supp Figure 4G). The available samples are randomly re-assigned into classes of equal number and size as the real data. The sPLS-DA pipeline (including selecting sPLS-DA parameters for the maximum number of components investigated and the number of features to include in each component) is applied to the randomised data, and the most accurate result recorded. This process is repeated 50 times. The estimated p value corresponds to the count of randomised accuracies surpassing that of the real data; 50 replicates yields a p value granularity of 0.02. Through this approach we gauge the potential for model overfitting – the likelihood that model accuracies reflect random chance or having identified sPLS-DA model parameters that serendipitously proffer superior performance despite the use of LOO-CV. This is an unavoidable when limited data are available and is evidenced by randomised data prediction accuracies greater than the e.g. 50% that would be expected when employing two classes.

The sPLS-DA ordinations show the two components capturing the most variance in the data; sPLS-DA models can include additional components (as indicated in figures). These ordinations represent the building of an sPLS-DA model on all available data using optimal

parameters as determined through LOO-CV. For taxa comprising signatures that distinguish experimental groups, we report only those taxa that were included in >90% of models built under LOO-CV; tables identifying employed in each sPLS-DA component are available at https://github.com/marknormanread/henstridge-2019. The experimental groups these taxa associate with are derived from the final model constructed over all available data (and reported in the ordinations).

Biochemical Analysis

Insulin: Insulin concentrations were measured with a mouse ultrasensitive Insulin ELISA (ALPCO Immunoassays, NH, USA). Plasma samples were read in a 96-well plate on a FLUOstar Omega microplate reader (BMG Labtech, VIC, Australia). Absorbance was measured at a wavelength of 450 nm.

Lipopolysaccharide Binding Protein (LBP): LBP was measured via ELISA with the absorbance measured at 450nm with a spectrophotometer (Hycultec, Beutelsbach, Germany).

Complete blood count. For hematological assessment, 20μL of whole blood was diluted 1:7 in Sysmex CELLPACK™ (Sysmex, Japan) diluent and assessed using an automated hematology analyzer (Sysmex XS-1000*i*, Kobe, Japan).

TAG Assay: Tissue TAG content was quantified using a colorimetric assay kit and calculated as μg/mg tissue (Triglycerides GPO-PAP; Roche Diagnostics, NSW, Australia).

Statistical Analysis

Metabolic data were analysed by three or two-way analysis of variance (ANOVA) and Tukey post-hoc tests when there was a significant interaction. All data are presented as mean ± standard error of the mean (SEM). Statistical significance was tested at $p<0.05$. Microbiota data was analysed as described above.

RESULTS

Metabolic characteristics of donor mice.

Donor mice were indistinguishable for body weight and lean mass at baseline (Fig 4A,C), however there was slightly less adiposity in the groups later designated for ET (Fig 4B, D). After 4 wk on their respective diets, the HFHS groups had greater body weight, fat mass and fat mass percentage and less lean mass compared with NC (Fig 4E-H). Relative to Sed, ET resulted in lower body weight, fat mass, lean mass and fat mass percentage (Fig 4I-L). ET led to improvements in exercise capacity on both diets, with mice on NC out-performing mice on the HFHS diet (Fig 5A). HFHS-fed mice exhibited higher daily caloric intake, but ET (vs Sed) proved inconsequential (Fig 5B). The HFHS diet caused glucose intolerance and ET countered it (Fig 5C), likely due to improved insulin sensitivity given there was no ET effect in basal or glucose-induced insulin secretion (Fig 5D,E). RER values were lower in mice fed a HFHS diet, but ET had no effect (Fig 5F). Summarily, the donor mice from which fecal material were collected for FMT displayed increased adiposity, glucose intolerance, caloric intake and hyperinsulinemia in the HFHS groups, with ET reducing adiposity and improving exercise capacity and decreasing glucose levels during an oGTT.

FMT recipient mice do not display changes in body composition.

Prior to commencing FMT, baseline body composition was assessed in the recipients with no difference observed for body weight, fat mass, lean mass or fat mass percentage (Fig 6A-D). The FMT treatment period had no effect on the recipients' body composition with body weight, fat mass, lean mass and fat mass percentage not different between groups in the week of the last gavage (Fig 6E-H) or at the end of the study ~3wks later (Fig 6I-L). Consequently, any alteration in microbiota delivered to the recipients did not impact body composition and any subsequent effect was independent of adiposity. The potential exists for microbiota to

influence feeding behaviour (22) however, consistent with the body composition data, no change was detected in recipient intakes (Fig 7A).

FMT recipient mice display higher glucose levels when challenged when treated with HFHS-donor microbiotas.

Interestingly, despite the lack of an adiposity or food intake phenotype, mice administered FMT from HFHS donors, irrespective of donor ET status, had a spike in their blood glucose levels at early timepoints following an oGTT (Fig 7B), suggesting a defect in the ability to clear glucose or inhibit endogenous glucose production. Calculation of incremental AUC (iAUC) for the glucose curve demonstrated a significant increase in the mice receiving HFHS-FMT out to 90 minutes (Fig 7C) that was reduced to a trend by 120mins ($p=0.081$, Fig 7D). As higher glucose levels could result from a pancreatic phenotype (if less insulin is secreted), we assessed basal and glucose-stimulated insulin secretion (15-minute) when the greatest difference in glucose was observed in the oGTT. There was no difference between the groups at either timepoint (Fig 7E,F). As insulin levels were unchanged, we next assessed whether there was a difference in insulin-stimulated glucose clearance into tissues. While we found no change in insulin-stimulated glucose clearance into skeletal muscle or brown adipose tissue (Fig 7G, H), we did detect a decrease in glucose clearance into epididymal white adipose tissue (WAT) in recipients of FMT from HFHS-donors (Fig 7I). We also assessed sections of the GIT, relevant given FMT, finding a decrease in the glucose clearance into the large but not small intestine under HFHS-donation (Fig 7J,K).

Glucose tolerance defect in HFHS-R mice is not due to lipid accumulation, physical activity or energy expenditure.

Given the alteration to glucose levels during the oGTT in the HFHS recipient mice, we assessed possible mechanisms of action. Despite no difference in adiposity, it is possible that

there had been alterations to the partitioning of lipids within organs which can disrupt glucose metabolism. We assessed triacylglyceride levels in the large intestine and WAT (locations of insulin-stimulated glucose clearance defects), but found no difference between recipient groups (Fig 7L,M). As hepatic steatosis is linked to disrupted glucose handling (42), we also measured triacylglyceride levels in the liver, but again observed no difference (Fig 7N).

It is possible that alterations to physical activity, substrate utilisation or energy expenditure could drive a change in glucose disposal before such changes manifest in the body composition data. Indirect calorimetry studies could not detect a difference in oxygen consumption, energy expenditure or physical activity levels between the groups (Fig 8A,B,D). There was a decrease in the RER in the NC-Ex-R group (Fig 8C), suggesting a preference towards fatty acid utilisation, however, we reiterate, a decrease in triacylglyceride content was not evident in the three tissues we assessed (Fig 7J-L). This finding of a decrease in RER in the NC-Ex-R group was independent of donor RER given there was not a difference in donor RER with exercise on the NC diet (Fig 5F).

Glucose tolerance defect in HFHS-R mice is not due to leaky gut, or changes to blood profile or inflammation.

A well characterized mechanism of diet-induced change in host-microbiota interaction is the inflammophile positive feedback loop, wherein microbes capable of anaerobic growth on inflammatory factors promote inflammation to gain a competitive ecological advantage (16). To assess this, we measured gut permeability and lipopolysaccharide-binding protein (LBP), an acute phase protein produced by the liver that recognises lipopolysaccharide. We detected no difference between recipient groups under either measure (Fig 8E,F). Finally, we conducted a complete blood count to determine any effect of the FMT on circulating cells. White blood cells, platelets and red blood cells were all unchanged in the recipients (Fig 8G).

The microbiotas of HFHS-R mice are distinguishable from NC-R mice with limited effect of ET status.

We next profiled the gut microbiotas of recipient mice via fecal 16S ribosomal RNA sequencing before and after their FMT, confirming that each donor intervention induced distinct and transmissible microbial communities, and seeking a potential explanation for the elevated glucose during the oGTT. Recipient mice microbiotas were dominated with members of the *Bacteriodetes* and *Firmicutes* phyla, (Fig 9A,B). We discounted insufficient or differential sequencing depth as confounding factors for subsequent analysis (Fig 9C,D). As expected, pre-FMT microbiotas exhibited no clustering by experimental group, (Fig 9E). However, post-FMT microbiotas did cluster by donor diet, but not donor exercise state (Figs 9F, Fig 10A,B). Microbiota ecological diversities also differed with donor diet (Fig 10C); diversity was higher in recipients of NC-donors, and highest in the NC-ET-R group. In summary, donor diet explained much of the variation between samples and lead to less diverse microbiota communities in the HFHS-recipients; this was not so for donor exercise state.

Next, we sought signatures, comprising subsets of taxa, in recipient microbiotas that were distinguishing of donor diets and exercise states through supervised machine learning (Fig 10D). We derived a model representing signatures correctly indicative of sample donor group (NC-Sed, NC-ET, HF-Sed, HF-ET) on average 80% of the time (Fig 10E). For context, as a baseline, 'guessing' the majority group for all samples would yield an accuracy of 29% (majority group is HF-Ex, representing 10 of the total 34 samples). We sought to explicitly test and discount model overfitting as a driver of accuracy; the attained 80% exceeded all 50 applications of an identical methodology to random experimental group reassignments amongst these samples, indicating $p<0.02$ (Fig 11). The model was relatively complex, encompassing 35 unique amplicon sequence variants (ASVs) to distinguish the 4 groups

406 (Supp Table 1). Interestingly, distinct strains of identical phylotypic classification associated
407 with multiple experimental groups, suggesting strain-level functional differences. Examples
408 include strains classifying as *Erysipelotrichaceae Allobaculum*, *Bacteroidales*
409 *Muribaculaceae* and *Lachnospiraceae*.

410 Microbial signatures reliably indicative of donor diet, irrespective of donor exercise-state,
411 were also derived (Figs 10F, 11, Supp Table 2). ASV classifying as *Erysipelotrichaceae*
412 *Allobaculum* and *Bacteroidales Muribaculaceae* were over-represented in FMT recipients of
413 NC-fed donor. Interestingly, other ASV also classifying as *Bacteroidales Muribaculaceae*
414 were over-represented in the HFHS-R, further suggesting strain-level functional differences.
415 ASVs classifying as *Cyanobacteria 4C0d-2 YS2* were likewise over-represented in HFHS-R,
416 as was a *Lachnospiraceae* of undetermined genus.

417 Whilst we found experimental groups receiving ET and Sed FMT on the same diet to be
418 statistically indistinguishable at the whole microbiota-level (Fig 10C), machine learning does
419 identify microbial signatures indicative of donor exercise state (Figs 10D,G, 11, Supp Table
420 3), though not as robustly as for donor diet (Fig 10F). Associated with the FMT of sedentary
421 donors were ASV classifying as: *Lactobacillaceae lactobacillus*, *Alcaligenaceae sutterella*,
422 *Bacteroidales Muribaculaceae* (genus undetermined), *Prevotellaceae prevotella,*
423 *Lachnospiraceae* (genus undetermined). Associating exercised donors were ASV classifying
424 as: *Alphaproteobacteria RF32* (genus undetermined), other *Bacteroidales Muribaculaceae*
425 (genus undetermined), *Porphyromonadaceae parabacteroides*.

426

427

DISCUSSION

Altered gut microbiota composition has emerged as a component of the ET state. Indeed, recent studies even suggest that FMT could be used to increase endurance exercise performance (39). The precise role these changes have on host metabolism are largely unknown and are difficult to characterize given the multiple mechanisms through which exercise alters metabolic processes. Whether these changes are metabolically protective in an environment of over-nutrition is of interest, given the worldwide need to offset the growing incidence of obesity, insulin resistance and T2D. Through FMT, we investigated if exercise conferred microbiota-mediated effects of metabolic value to the host. While we confirm in non-germ-free, non-antibiotic treated mice that FMT from HFHS-fed donors is, at least in part, capable of negatively impacting recipient glucose levels during an oGTT, we find no evidence that FMT from ET donors alters recipients' metabolic profile.

Transfer of gut microbiota to recipient mice caused a glucose spike during an oGTT if the donor was fed a HFHS-diet, independently of donor exercise status. To put the magnitude of the effect into perspective, the average oGTT blood glucose difference at the 15 min mark between the NC-Sed and HFHS-Sed donor groups was 3.78mmol/L (Fig 5C). The difference at the same 15 min mark between FMT recipients of NC-Sed and HFHS-Sed was 2.81mmol/L (Fig 7B). Hence, contrasted with mice fed a HFHS diet for ~2months, simple FMT from these animals into NC-fed recipients transfers over 70% of the early glucose level defect. This finding is of interest given: 1) the recipient mice experienced no increase in adiposity, so it was not an adipose-driven phenomenon, 2) it was independent of a leaky gut-inflammatory environment and 3) the HFHS-Ex-R group phenocopied the HFHS-Sed-R group meaning that donors being exercised, having reduced adiposity and improved glucose levels during an oGTT, conferred no protective effect. This suggests that the "Fat but Fit

paradox" may not extend to the gut microbiota and associated host glucose metabolism regulation.

An important factor in identifying host-microbe metabolic interactions is exposure timing. Foley and colleagues (14) demonstrated that mice exposed to feces from soiled cages from high fat-fed mice in the short-term (4 days) had similar glucose tolerance as compared to mice exposed to feces from chow-fed mice. However, after longer-term (45 days) of microbiota exposure, mice that were exposed to feces from high fat-fed mice were more glucose intolerant compared to mice that received feces from chow-fed mice. Importantly, this is the same timeframe in which we assessed glucose control in our study. Given that this length of exposure time was significant enough to promote dysglycemia in both these studies in response to high energy-diet FMT/fecal exposure, we believe it is also long enough for any exercise-induced effects to manifest, though we cannot exclude that longer exposure time to the exercise-trained microbiota may be necessary to see an impact on glycemia.

One metabolic parameter that was assessed whereby there was an exercise effect on the recipients was the RER values for the NC-ET-R group which showed lower values compared to the NC-Sed-R group (Fig 8C). A lower RER is indicative of a drive towards fatty acid utilisation/oxidation and linked to endurance training due to mitochondrial enzyme adaptations. However, in our hands we saw no difference with exercise training in the donors in relation to RER (Fig 5F. Consequently, it wasn't the case of a direct effect of the NC-ET donors having a lower RER and transmitting that to the recipients and must have arisen due to more complicated mechanisms. While we saw no effect on TAG levels in the large intestine, WAT or liver of the NC-ET-R group, we cannot exclude the possibility that there were lipid alterations in other organs. The effect was only observed in the NC groups suggesting that its transmission was nullified when the donors were fed a HFHS-diet. Future studies designed to specifically elaborate on this finding may be warranted.

Our data also demonstrate that diet asserts dominance over physical activity in relation to gut microbial composition and penetrance of recipient metabolic phenotypes with FMT. In untargeted analyses recipient microbiotas readily clustered by donor diet but donor exercise state was indistinguishable. Whilst targeted supervised machine learning did identify subsets of microbes that reliably identified exercise status, the models were more nuanced and encompassed a greater number of microbes than when distinguishing donor diet. This suggests that donor diet was readily determinable through few microbes, but exercise induced far more subtle patterns spanning multiple microbes.

During the preparation of this manuscript a paper was published (26) whereby the authors performed a similar set of experiments. In their study, administration of FMT from ET mice to antibiotic-treated high fat-fed recipient mice (5 FMT's/wk for 12 wk) decreased adiposity and blood glucose levels in the recipients (26). While this previous study suggested exercise conferred some microbiome-mediated effects, they were predicated on a fairly extreme protocol of treatment of antibiotics and many gavages. Our study suggests that such effects would be relatively minor under more realistically tempered contexts of people adhering to regular physical activity. The discordant results of their study with our own likely stem from methodological differences. The current study used NC recipients while the previous study used high fat-fed (60% fat) recipients. We avoided antibiotic pre-treatment whereas their mice received ciprofloxacin/metronidazole. The present study used 6 FMT's where the other study performed 60. Also, we utilised a sham gavage control group (NC-Sed donors → NC-Sed recipients) to control for 1) the stress of gavage treatment and for any potential metabolic impact of this and 2) introducing new microbial constituents to the gut; conversely, the control group in the other paper was treatment naïve (26). It would be interesting in future studies to combine their approach with our method. That is, to test the effect of FMT from ET donors on high-fat-fed recipients but at less frequency, with the inclusion of a gavage control

group and with and without antibiotic treatment. Another additional experimental design may be to test if precolonization of recipient mice with microbes from ET-donor mice could protect against the effects of later, subsequent exposure of FMT from HFHS-fed mice.

The specific composition of high caloric diet may also limit whether exercised microbiota is transmissible and/or metabolically protective as microbiota transmissibility has been previously shown to be non-effective when recipients are fed a diet high in fat (37). Consequently, looking at our study and other similar studies, the pathological or physiological metabolic state of the recipient may be an important determining factor in the effectiveness of FMT or the ability of FMT to assert a phenotype. This is an important factor for scientists to consider when conducting such studies.

Our study has some limitations worthy of consideration. Despite investigating numerous potential mechanisms via which the FMT from HFHS-donors may be causing the glucose spike during the oGTT in recipients (i.e. blood cell profile, leaky gut, lipid accumulation, inflammation, microbiota composition) we cannot provide a precise mechanism of action. Other mechanistic candidates for the effect include, but are not limited to, tissue cross-talk signalling, neural regulation, alteration to blood flow and glucose transport regulation. Additionally, as our oGTTs did not contain glucose stable isotope tracers we could not determine the relative contribution of the liver to the glucose spike phenotype. The study assessed only one method of delivering the FMT (orally). It is possible that rectal FMT may have yielded a different profile of viable microbes colonising the gut, and thus a differing metabolic phenotype. We also cannot conclude whether the observed effects were due to the microbiota changes or other fecal-derived components that were transferred, or whether the effects require microbial engraftment, as gavages were administered periodically throughout.

Determining the ratio of alive/dead bacteria that is transplanted with FMT in future studies may provide information in determining factors impacting on glucose metabolism following FMT. Further, completely recolonising the gut with FMT (i.e. like what occurs with the use of germ-free mice or broad-spectrum antibiotic treatment) may have yielded different results. Such procedures may promote the engraftment of microbes during and following on from the FMT process, when the intestine is devoid of microbiota and consequently the competition for engraftment and growth is lowered. We selected our approach to test our hypothesis with mice in their natural state to simulate as closely as possible the normal physiological system (i.e. a system where we tested FMT efficacy in mice that already harboured a natural microbiota composition; this would better reflect the microbiota of people commencing an exercise regime with diet-induced microbiota in place).

Together, our study demonstrates that the negative effects of HFHS-diet on glucose levels during an oGTT can be transferred to recipient mice via FMT. This may be an important factor to consider when screening donors for FMT. Indeed, there are case reports of potential transmission of new-onset obesity via FMT (1), so donor-metabolic screening may need prioritisation to protect the metabolic health of recipients. Our study also questions whether ET-induced changes in microbiota composition can dominate over normal diet-influenced composition to an extent to alter host metabolism as besides an alteration to RER (on normal chow diet only), no metabolic phenotype was observed in recipient mice.

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Conflicts of Interest:

None declared.

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574 ## References

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712 **Figure Legends**

713 **Fig 1:** Flow diagram of animal use and analysis for the donor groups based on the
714 Consolidated Standards or Animal Experiment ReporTing (CONSAERT) template.

715

716 **Fig 2:** Flow diagram of animal use and analysis for the recipient groups based on the
717 Consolidated Standards or Animal Experiment ReporTing (CONSAERT) template.

718

719 **Fig 3:** Study design for donor and recipient groups.

720

721 **Fig 4.** Body composition analysis in fecal donor cohort. A-D) Body weight, fat mass, lean
722 mass and body fat percentage at baseline (7-8 wk of age), following 4 wk of dietary
723 intervention (pre-exercise) (E-H) and following ET intervention (I-L). NC-Sed n =17, NC-ET
724 n=20, HFHS-Sed n=17, HFHS-ET n=21. 2-way ANOVA. ** p = <0.01, *** p = <0.001 for
725 dietary main effect, # p =<0.05, ## p = <0.01, ### p = <0.001 for ET main effect.

726

727 **Fig 5.** A) Exercise capacity from an incremental exercise test pre and post training, NC-ET
728 pre and post n =11, HFHS-ET pre and post n = 10. B) Caloric intake per day during training
729 period (measured and averaged per 24 h over 4 days, n =4) C) Glucose excursions from an
730 oral glucose tolerance test (oGTT), NC-Sed n= 9, NC-ET n=11, HFHS-Sed n=10, HFHS-ET
731 n=10. D,E) Plasma insulin levels at baseline and 15 min into the oGTT, NC-Sed n=9, NC-ET
732 n=11, HFHS-Sed n=10, HFHS-ET n=10. F) RER of donor cohorts as measured in metabolic
733 caging experiments. n=6 per group. 2-way ANOVA. * p = <0.05, *** p = <0.001 for dietary
734 main effect, # p =<0.05, ## p = <0.01, ### p = <0.001 for ET main effect.

735

736 **Fig 6.** Body composition of FMT recipient mice. A-D) Body weight, fat mass, lean mass and
737 body fat percentage at baseline (7-8wks of age), following 6wks of weekly FMT intervention
738 (E-H) and at endpoint of study ~3wk after last gavage (I-L). NC-Sed-R n =13, NC-ET-R
739 n=15, HFHS-Sed-R n=14, HFHS-ET-R n=16.

740

741 **Fig 7.** Metabolic characteristics of FMT recipient mice. A) Food intake per day during
742 gavage period (measured and averaged per 24 h over 4 days, n=4. B) Plasma glucose
743 excursion curves from an oral glucose tolerance test, NC-Sed-R n =13, NC-ET-R n=15,
744 HFHS-Sed-R n=14, HFHS-ET-R n=16. C-D) Incremental area under the glucose curve
745 analysis, NC-Sed-R n =13, NC-ET-R n=15, HFHS-Sed-R n=14, HFHS-ET-R n=16. E-F)
746 Plasma insulin concentration before and during the oral glucose tolerance test, NC-Sed-R n
747 =7, NC-ET-R n=9, HFHS-Sed-R n=8, HFHS-ET-R n=10. G-K) Insulin-stimulated glucose
748 clearance into G) skeletal muscle, H) brown adipose tissue (BAT), I) white adipose tissue
749 (WAT), J) large intestine, K) small intestine, NC-Sed-R n =7, NC-ET-R n=9, HFHS-Sed-R
750 n=8, HFHS-ET-R n=6. Triacylglyceride levels in L) large intestine, NC-Sed-R n =7, NC-ET-
751 R n=9, HFHS-Sed-R n=8, HFHS-ET-R n=6, M) white adipose tissue, NC-Sed-R n =5, NC-
752 ET-R n=5, HFHS-Sed-R n=4, HFHS-ET-R n=6, and N) liver, NC-Sed-R n =7, NC-ET-R
753 n=9, HFHS-Sed-R n=8, HFHS-ET-R n=6. 3 or 2-way ANOVA. * $p = <0.05$, ** $p = <0.01$ for
754 diet effect.

755

756 **Fig 8.** Metabolic measures in FMT recipient mice. A) $VO_{2,}$ B) Energy expenditure, C)
757 Respiratory exchange ratio (RER) and D) physical activity levels from metabolic caging
758 experiments, n=6/group. E) Plasma FITC-dextran measurement as a measure of gut
759 permeability, NC-Sed-R n=5, NC-ET-R n=3, HFHS-Sed-R n=5, HFHS-ET-R n=6. F) Plasma

LBP concentration as a marker of systemic inflammation, NC-Sed-R n =8, NC-ET-R n=10, HFHS-Sed-R n=10, HFHS-ET-R n=12. Plasma analysis in FMT recipient mice. G) Total white blood cells, platelets and red blood cell counts. n=6/group. 2-way ANOVA with tukey post-hoc for interaction (when between 2 bars). # p = <0.05, ### p = <0.001 for ET effect. ** p = <0.01, ***p=<0.001 for diet effect.

Fig 9. Fecal gut microbiota composition in recipient mice post-FMT. Microbiota composition of recipients at the A) phylum and B) family levels, based on bacterial 16S rRNA sequencing. C) Number of sequence reads per sample, collected by donor group. D) Rarefaction curves quantifying distinct microbes (amplicon sequence variants, ASV) identified when subsampling the available data; sequencing effort adequately captured the diversity of microbes present. Principal component analysis ordinations showing recipient microbiota similarities of recipients E) at baseline (pre-FMT) and F) following gavage treatment study conclusion. NC-Sed-R n =7, NC-ET-R n=9, HFHS-Sed-R n=8, HFHS-ET-R n=10.

Fig 10: Donor diets drive community-level differences in recipient microbiota, but both donor diet and exercise statuses induce distinguishing microbial signatures in subsets of taxa. A) Assessing the clustering of microbiotas by donor group by contrasting between- versus within-group Aitchison distances; distance distributions shown in B. C) Microbiota alpha diversity quantifications: the number of distinct amplicon sequence variants observed, Shannon and Inverse Simpson metrics. Black circles indicate mean values. * p = <0.05, ** p = <0.01, *** p = <0.001 for dietary effect; no ET effects found; assessed through PERMANOVA. D) We assess whether donor experimental statuses induce distinguishing alterations across subsets of recipient microbiota taxa ('signatures') through supervised

785 machine learning (sparse partial least squares discriminant analysis, sPLS-DA). Such
786 signatures can emerge even in absence of significant whole-community-level effects, as for
787 ET status. E-G) sPLS-DA models are constructed to distinguish recipients' microbiotas based
788 on E) donor diets and ET states, F) diets irrespective of exercise state and G) exercise states
789 irrespective of diet. NC-Sed-R n =7, NC-ET-R n=9, HFHS-Sed-R n=8, HFHS-ET-R n=10.
790
791 **Fig 11:** Microbial signatures derived through sPLS-DA were robust and not the result of
792 models overfitting to noise in the data, determined through repeated model re-training under
793 permuted data. All sPLS-DA models on real data (Fig 10E-G) were statistically significant.
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FIG 1

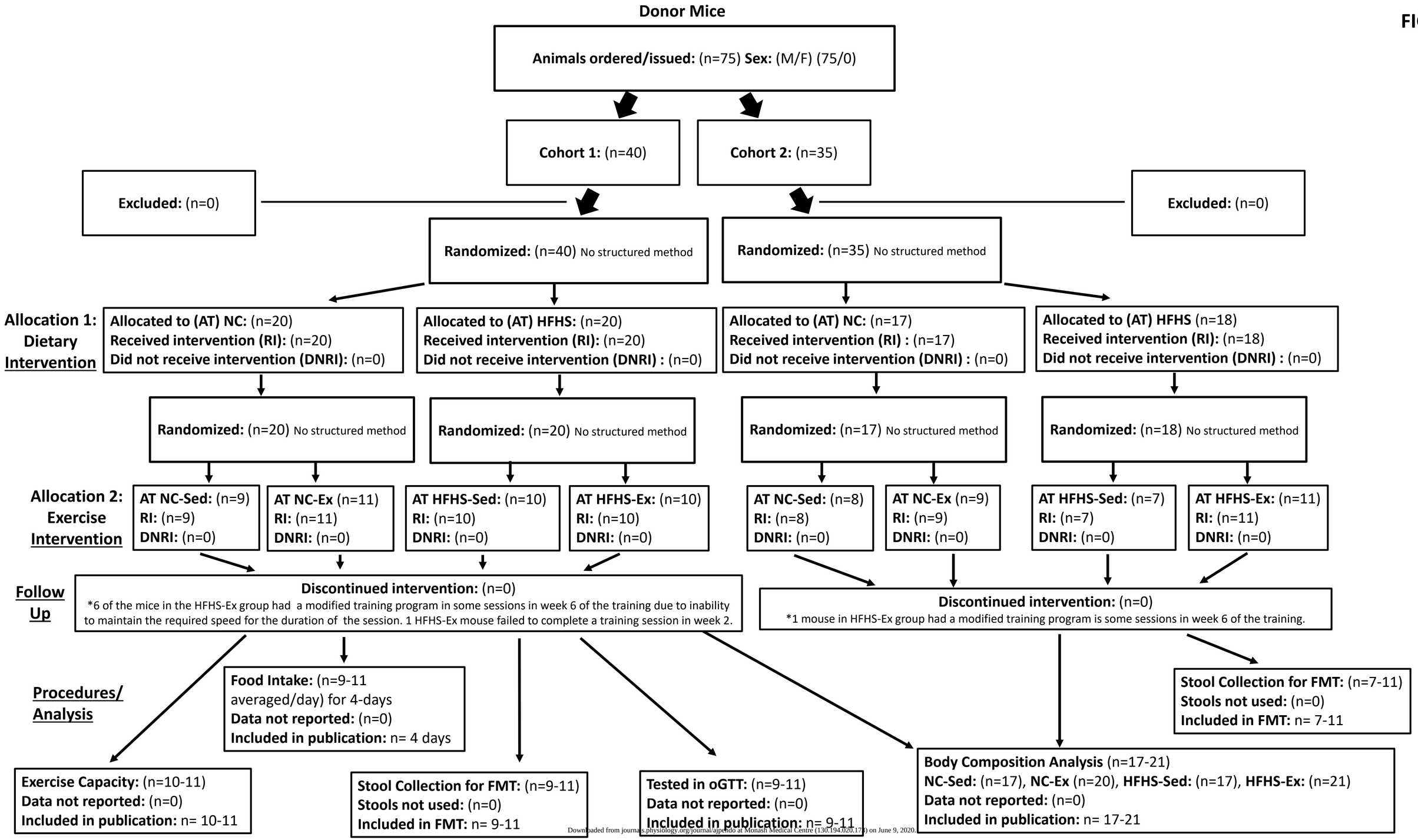


FIG 2

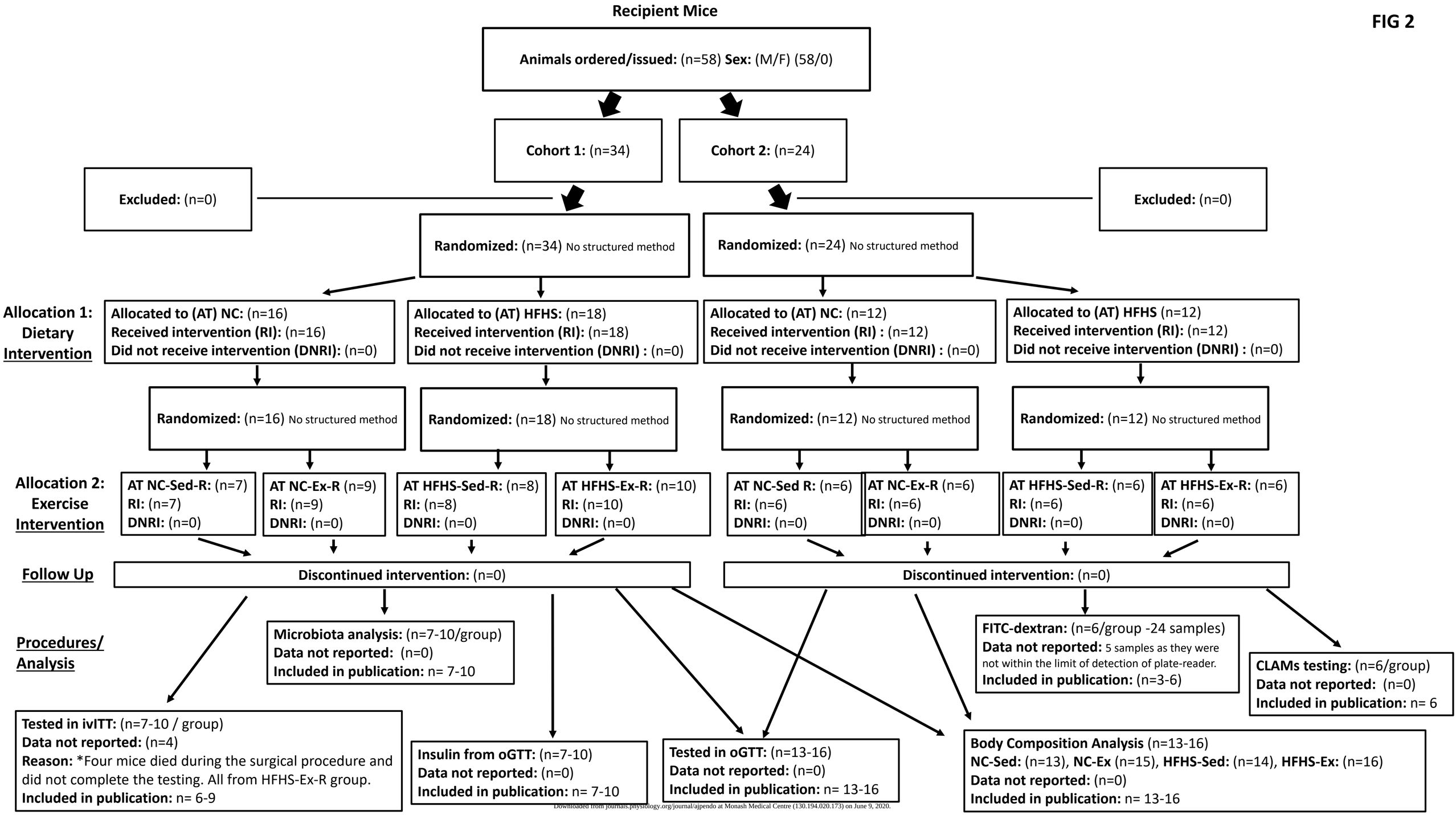


FIG 3

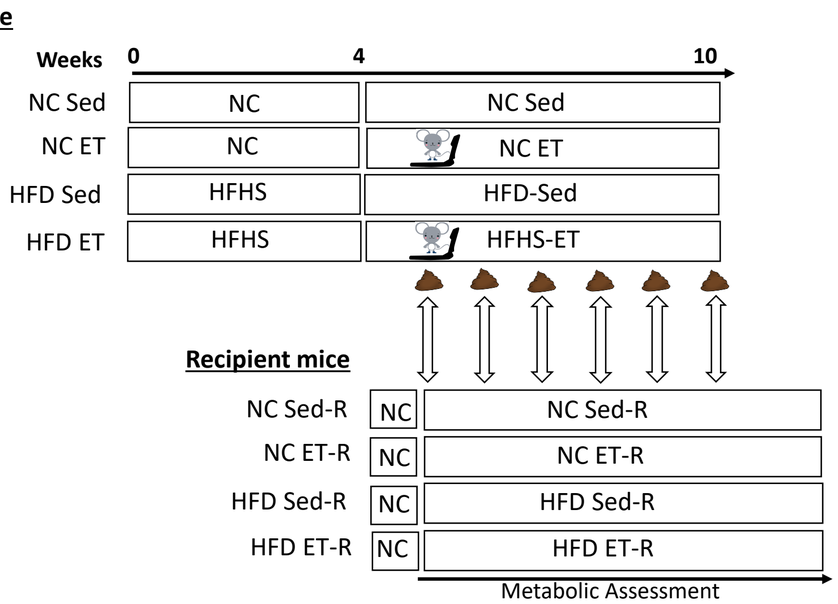
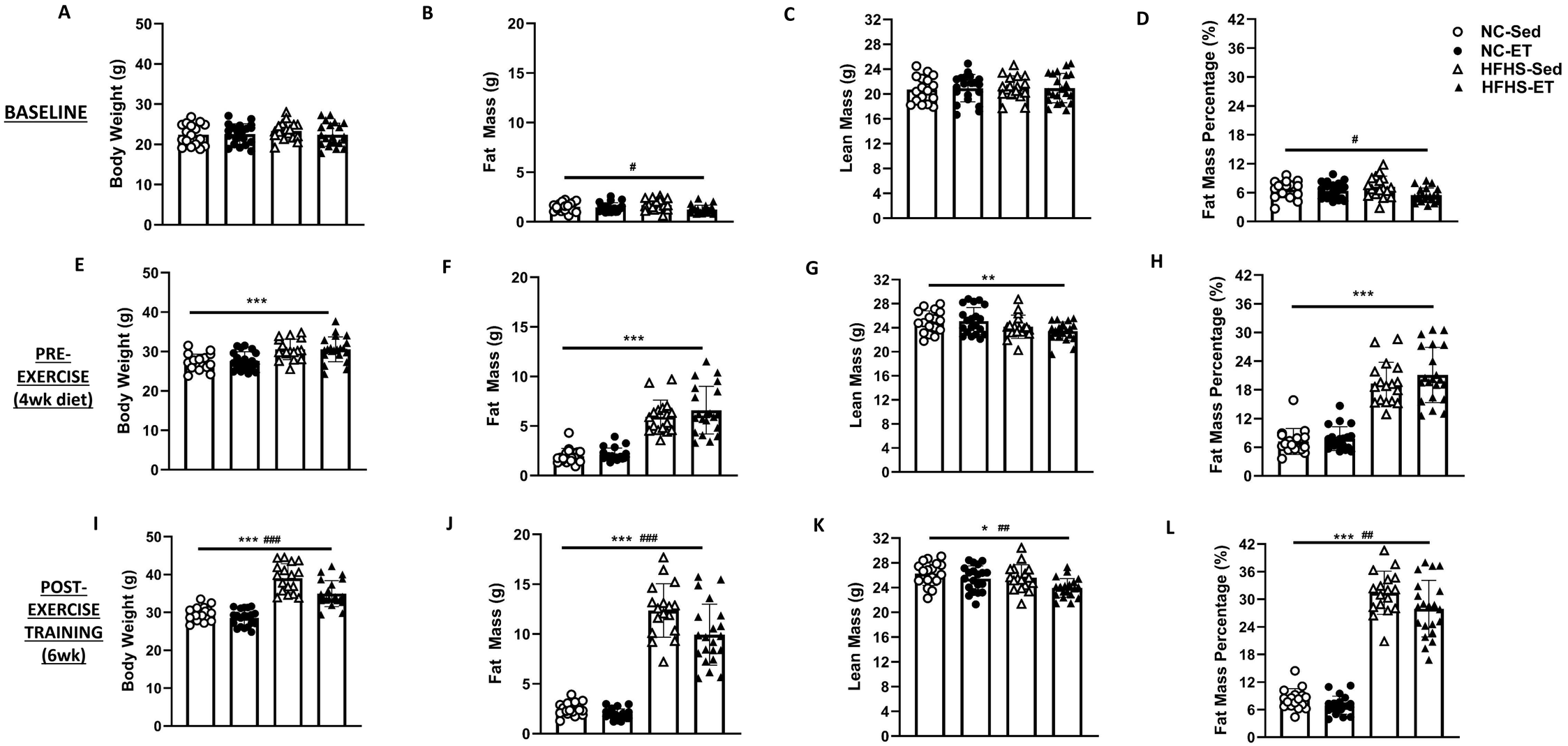


FIG 4



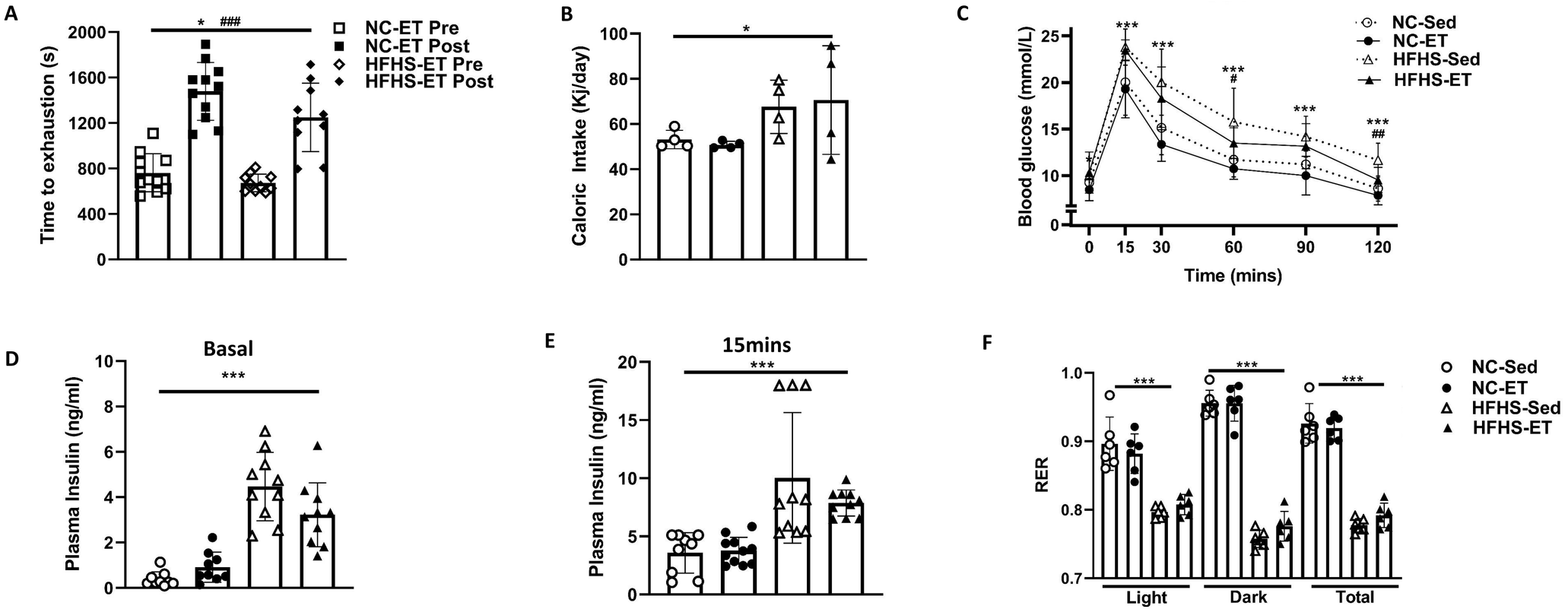
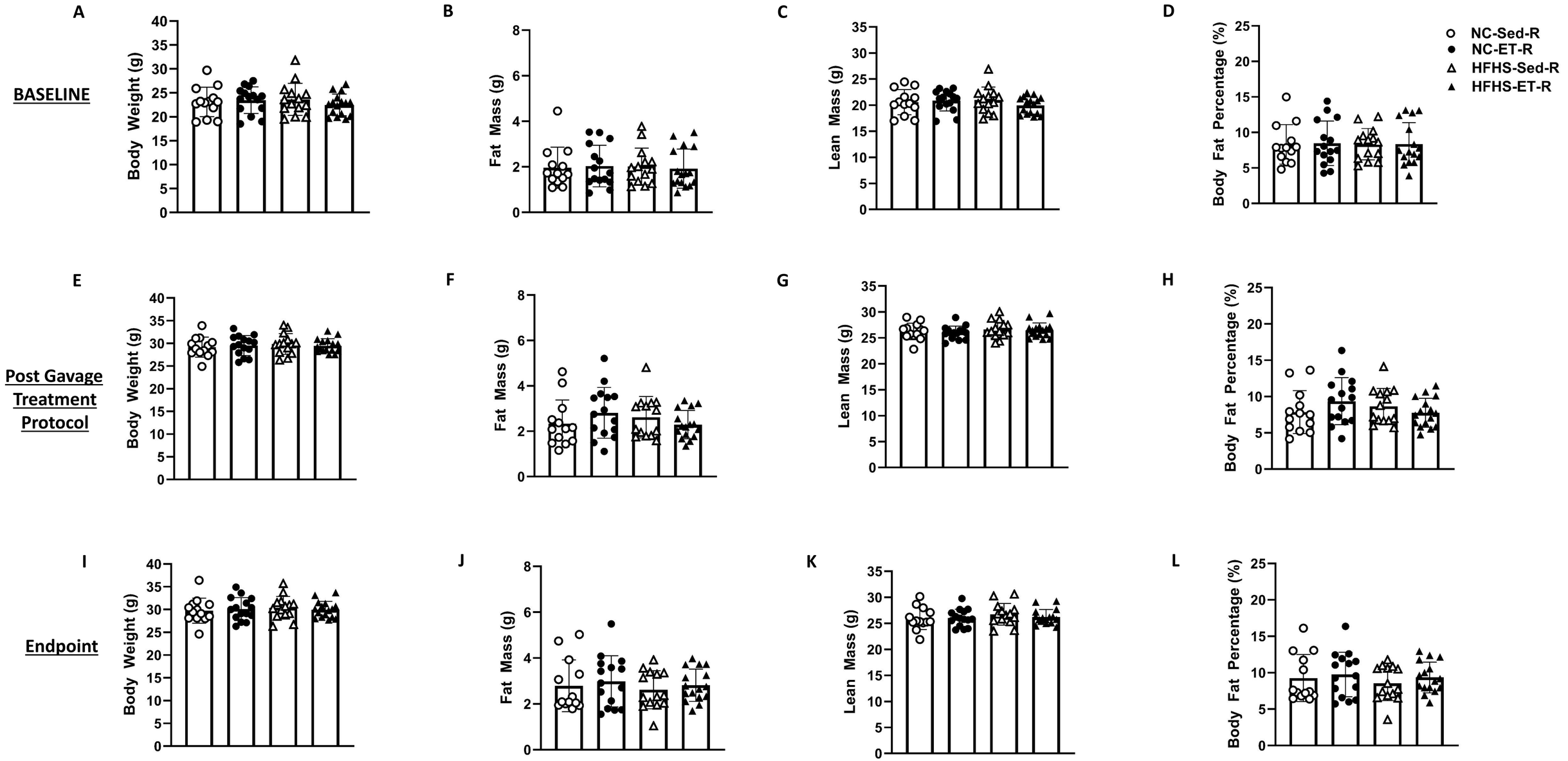


FIG 6



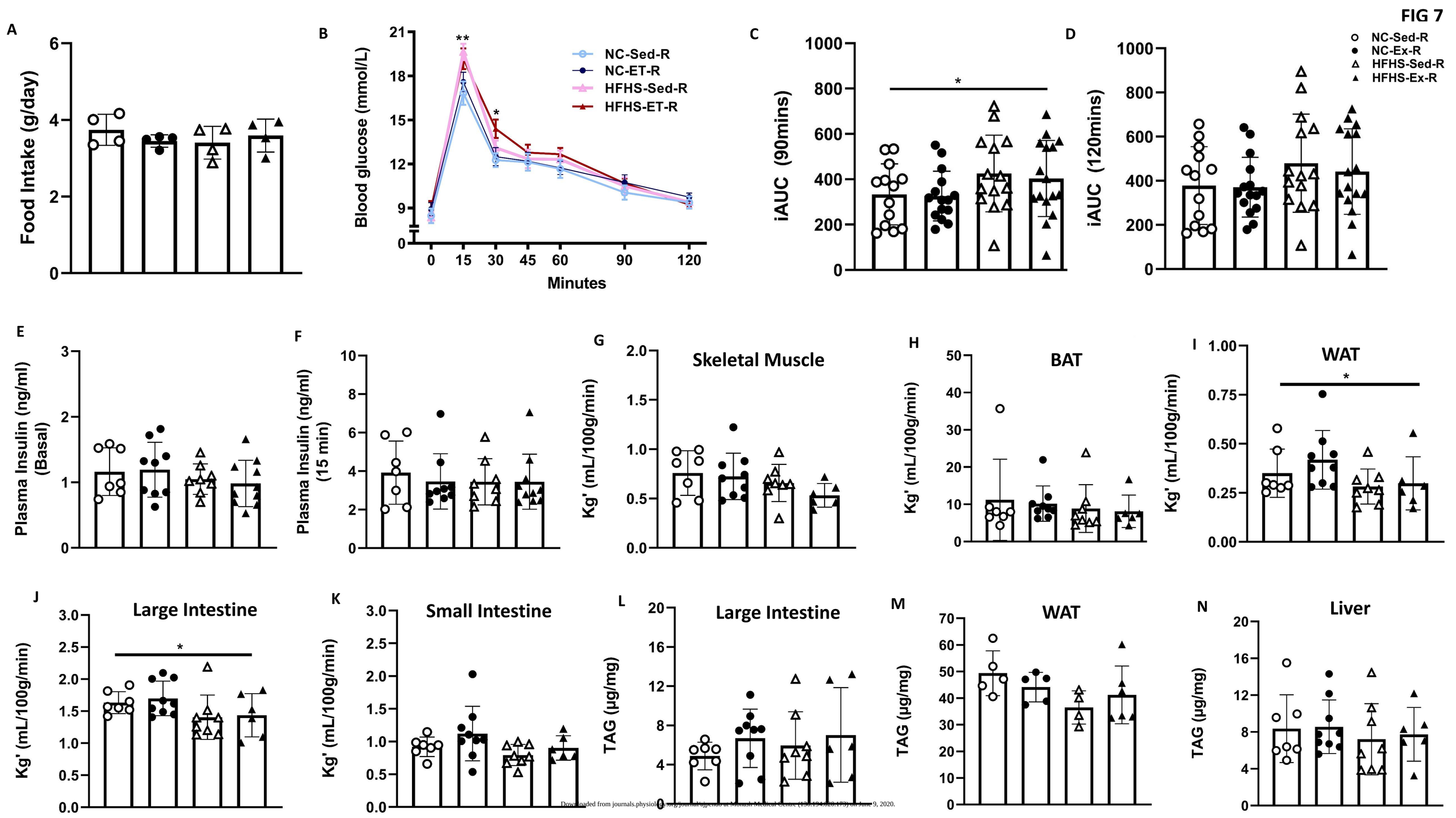
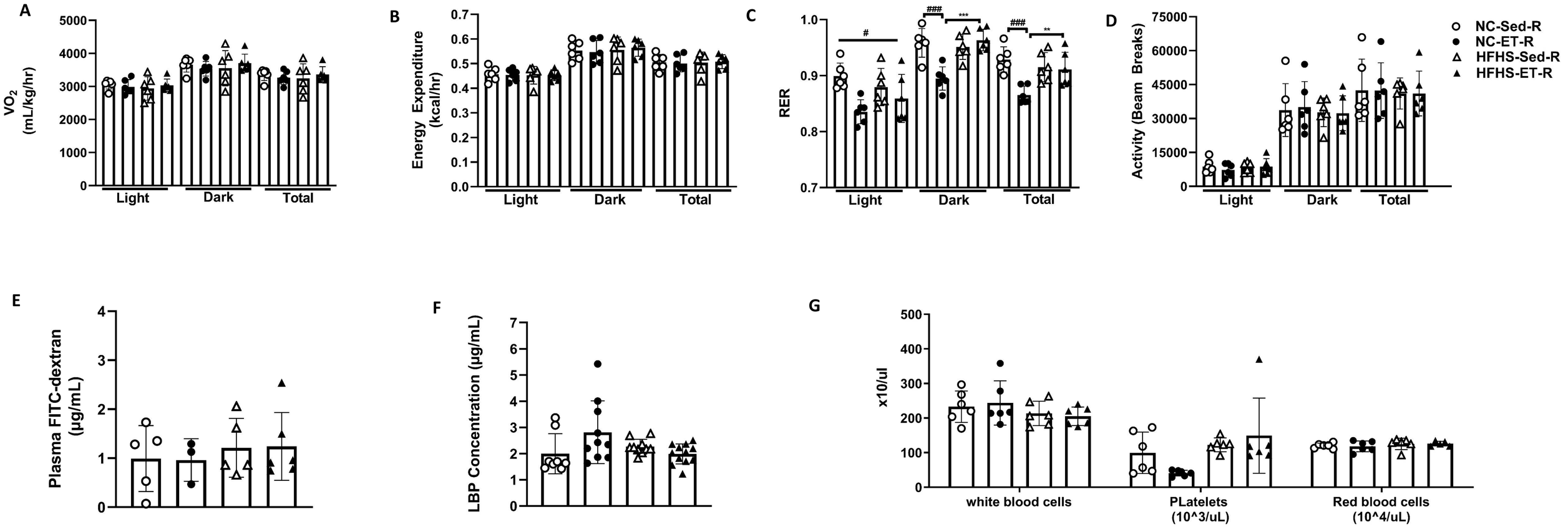
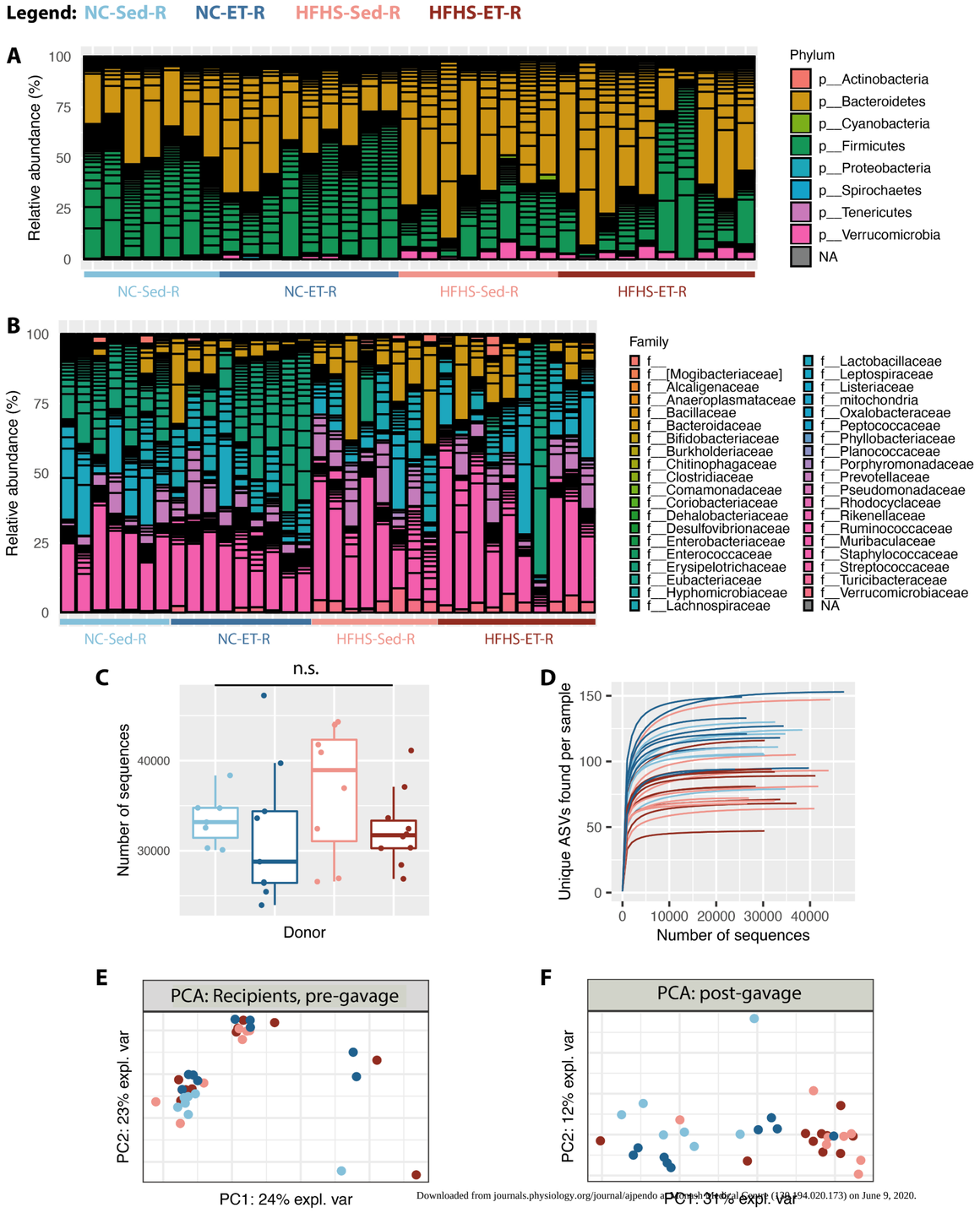
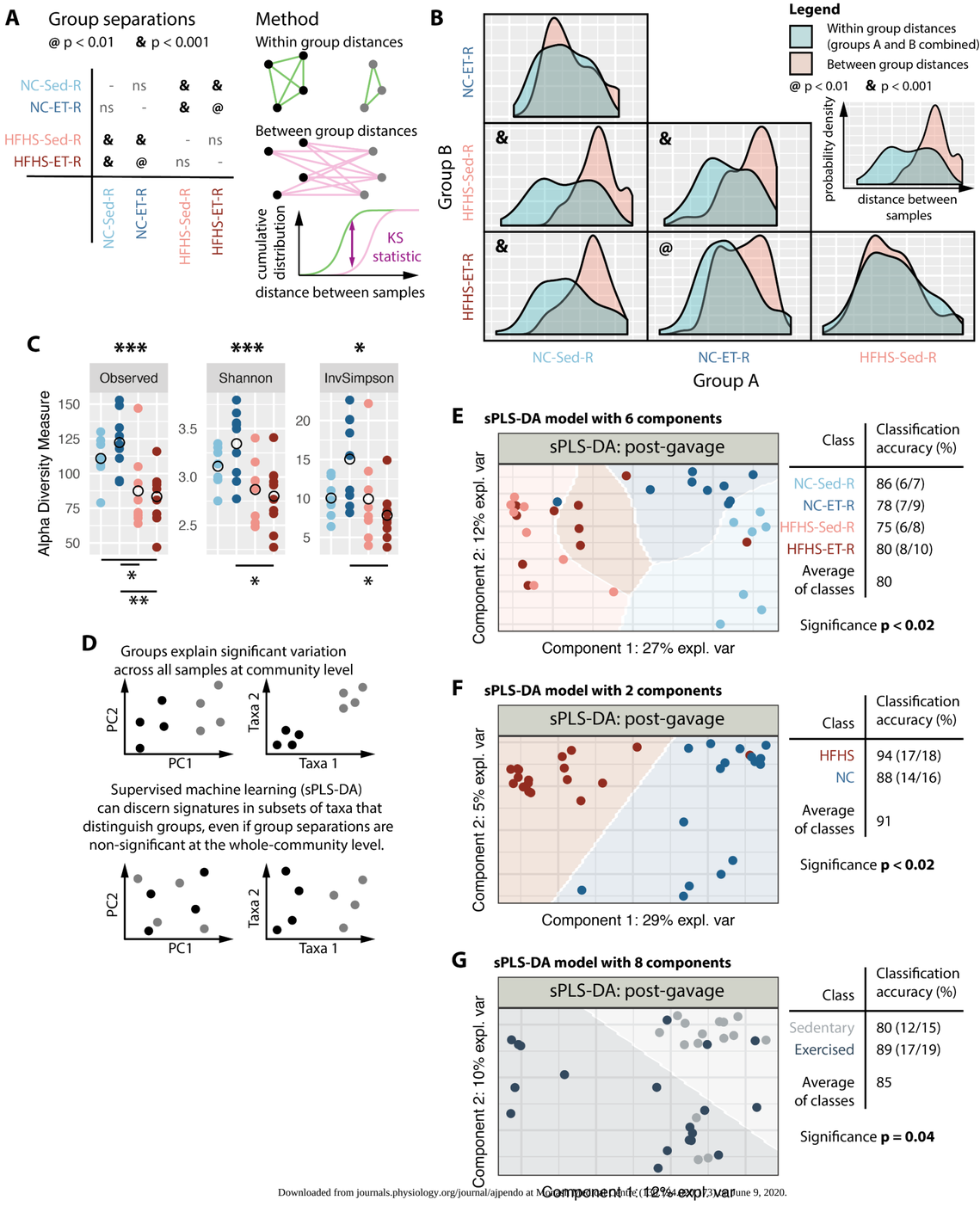


FIG 8





Legend: NC-Sed-R NC-ET-R HFHS-Sed-R HFHS-ET-R



Quantifying sPLS-DA model overfitting potential:
sPLS-DA model performances when learning on random group re-assignments, 50 replicates